Reprinted from the PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES
Vol. 58, No. 5, pp. 2043–2050. November, 1967.

FLUORESCENCE STUDIES OF THE INTERACTION OF NUCLEOTIDES WITH THE ACTIVE SITE OF THE NUCLEASE OF STAPHYLOCOCCUS AUREUS

BY PEDRO CUATRECASAS,* HAROLD EDELHOCH, AND CHRISTIAN B. ANFINSEN

LABORATORY OF CHEMICAL BIOLOGY AND CLINICAL ENDOCRINOLOGY BRANCH,
NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES,
NATIONAL INSTITUTES OF HEALTH, BETHESDA, MARYLAND

Communicated September 7, 1967

The fluorescence intensity (or quantum yield) of proteins affords a sensitive method of following the interaction of enzymes with their substrates, inhibitors, or other cofactors.[1] The fluorescence spectra of nonconjugated proteins are essentially determined by the contributions of the tyrosyl and tryptophanyl residues, with the latter usually predominating.[2] The fluorescence of phenylalanine has not been observed in proteins containing the two former residues.

The fluorescence intensities of the tyrosyl and tryptophanyl chromophores are affected by specific interactions[3] and by solvent or environmental factors.[4, 5] The peaks of tyrosine and tryptophan fluorescence are separated by about 50 mμ, and the tyrosyl peak is usually masked by the stronger emission of the tryptophanyl residue.[2] However, under favorable circumstances, the interactions of the protein can be monitored by measurements of the fluorescence of both residues. If the changes are not in unison, it is possible to monitor different regions of the molecule.

The DNase and RNase activities of the extracellular nuclease of *Staphylococcus aureus* are competitively inhibited by a number of nucleotides.[6] In the presence of Ca^{++} these substrate analogues bind to the active site of the enzyme in a one-to-one molar ratio.[7] As a consequence of the specific binding of deoxythymidine-3′, 5′-diphosphate (dTdiP)[8] to nuclease, three to four of the seven tyrosyl residues of the protein become unreactive to acetyl imidazole. In the present study, the specific binding of a competitive inhibitor has a profound effect on tyrosyl and is without effect on tryptophanyl fluorescence. To supplement the information obtained from the fluorescence properties of the native enzyme, fluorescence and polarization of fluorescence measurements of nuclease conjugated with dimethylamino naphthalene sulfonyl chloride (DNS-nuclease)[8] have also been made.

Experimental Procedures.—Materials and methods: Staphylococcal (Foggi strain) nuclease was purchased from Worthington Biochemical Corp. Highly polymerized salmon sperm DNA was obtained from Calbiochem, and yeast RNA from Sigma Chemical Co. dTdiP[8] was purchased from Calbiochem. 5′-GMP[8] was obtained from Schwarz BioResearch and DNS from Mann Chemical Co. DNase and RNase assays were performed spectrophotometrically.[6] Protein concentration was determined by measurements of absorbance at 277 mμ, using $E^{1\%}_{1\ cm} = 9.70$.[9] The molecular weight of the nuclease is 16,800.[10]

Ultraviolet fluorescence: Fluorescence spectra of nuclease were measured with a Turner model 210 spectrofluorometer. The fluorescence intensities of tyrosine and tryptophan were measured at 295 and 360 mμ, respectively; in most cases excitation was at 280 mμ. In all studies, the enzyme (0.1 mg/ml) was dissolved in 0.1 *M* NaCl, and the temperature was maintained at 24° unless otherwise stated.

Preparation of fluorescent conjugates: Thirty microliters of dye, DNS, dissolved in acetone (3 mg/ml), was added per milliliter of nuclease (0.5%) in 0.1 *M* sodium bicarbonate, pH 8.2, at 25°. After the solution had become clear (10–20 min), it was passed through a G-25 (fine) Sephadex column to separate the conjugated nuclease from free and adsorbed dye. The protein-containing

effluent was dialyzed for 24 hr. against 0.1 *M* NaCl. To ensure complete removal of unconjugated dye, the protein was in some cases passed again through Sephadex and precipitated several times by adding crystalline $(NH_4)_2SO_4$ to 100% saturation.

The degree of labeling was determined from the absorbancy at 340 $m\mu$, using 4200 as the molar extinction coefficient of the dye.[11] The number of conjugated dye molecules per mole of nuclease varied from 0.8 to 1.2 in these studies.

The conjugating procedure did not result in loss of DNase or RNase activities. Upon excitation at 335 $m\mu$, the wavelength of maximum fluorescence intensity of the DNS-nuclease was 530 $m\mu$.

Polarization of fluorescence: Measurements of polarization were made with a modified Phoenix light-scattering photometer as described elsewhere.[12] A Corning 5970 filter intercepted the unpolarized incident beam, and a Corning 3385 filter was placed before the entrance slit of the photocell. The vertically and horizontally polarized components of the fluorescent light were recorded. The polarization, P, is defined as $(I_v - I_h)/(I_v + I_h)$, where I_v and I_h, are, respectively, the vertical and horizontal components. The mean rotational relaxation times ($\rho_h^{25°}$) were computed by means of the Perrin equation:

$$\left(\frac{1}{P} + \frac{1}{3}\right) = \left(\frac{1}{P_0} + \frac{1}{3}\right)\left(1 + \frac{3\tau}{\rho_h}\right),$$

where P_0 is the limiting polarization obtained by extrapolation of the plot of $(1/P) + (1/3)$ as a function of T/η. The excited lifetime of the dye conjugated to the protein, τ, was measured by the nanosec flash technique, with a modification of the TRW instrument's fluorescence decay time apparatus as described by Chen *et al.*[13] The measured value of 13 nanosec is similar to that previously described.[12] Assuming that the nuclease has the form of an unhydrated rigid sphere, a relaxation time, ρ_0, can be computed from knowledge of the molecular weight, 16,800, and a partial specific volume ($\bar{v}$) of 0.72[14]: $\rho_0^{25°} = 3\eta V/RT = 11 \times 10^{-9}$, where $V = M\bar{v}$, and η is the viscosity of water at 25°.

The Perrin equation assumes that the protein is randomly labeled. This was verified by a peptide map of a tryptic digest of DNS-nuclease. This showed a peptide pattern identical to

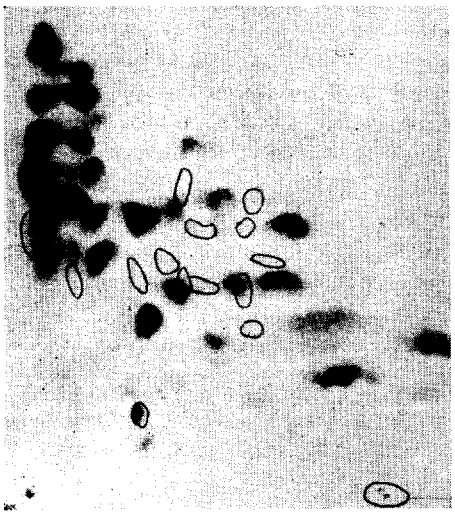

FIG. 1.—Peptide map obtained from a tryptic digest of DNS-nuclease. The digest, containing 1.5 mg of protein, was applied to Whatman no. 3MM paper for two-dimensional separation. Dark spots are ninhydrin-stained peptides, and encircled areas represent fluorescent peptides.

that of unconjugated nuclease, and at least 14 fluorescent areas were detected (Fig. 1). The latter did not correspond to areas of ninhydrin staining, which would have been expected if labeling had occurred at the epsilon amino groups of the lysine residues. The large number of fluorescent spots in amounts small enough to avoid detection by ninhydrin suggests that labeling probably occurred in nearly random fashion.

Results.—Ultraviolet fluorescence of native nuclease: The addition of dTdiP to a

solution of the nuclease results in a decrease in its fluorescence spectrum in the region of tyrosyl fluorescence and is without effect in the region of tryptophan emission. The fluorescence intensity at 295 mμ falls with the addition of dTdiP, as seen in Figure 2. An approximate dissociation constant of 4×10^{-7} M was calculated from the data. This value is about the same as the value obtained for dTdiP by the kinetics of inhibition of DNase and RNase activities,[7] gel filtration measurements,[7] and ultraviolet absorption difference spectroscopy.[15]

The effect of pH on the fluorescence intensity of nuclease in the absence of dTdiP is depicted in Figure 3. In the presence of dTdiP, there is a marked fall in tyrosyl fluorescence in the pH range from 5.5 to 10. The merging of the two curves between pH 7.0 and 5.0 presumably reflects the pH dependence of the dissociation of the nuclease-nucleotide complex. The red shift in tyrosine absorption which occurs with dTdiP binding shows a similar pH dependence.[15] Since the conformation of the enzyme appears to be essentially constant between pH 7.0 and 5.5 (upper curve, Fig. 3), the ionization of a charged group of the protein with a pK in this region (or of a phosphate group in the nucleotide) may be responsible for the dissociation of the inhibitor. The fall in tyrosyl fluorescence above pH 8 (Fig. 3) is due to the ionization of the tyrosyl residues.[15] In the presence of inhibitor, the quenching of tyrosyl fluorescence is shifted to higher pH values in harmony with a similar displacement found in the tyrosyl ionization curve.[15]

The quenching of tyrosyl fluorescence by dTdiP is not observed if Ca^{++} is omitted. Addition of 5′-GMP,[8] a nucleotide which does not inhibit enzymatic activity[6] and is poorly bound to the nuclease,[7] has no effect on nuclease fluorescence. The nucleotide, 5′-dTMP,[8] which binds to nuclease less well than dTdiP, produces, at a molar ratio of one, pH 7.5, one-half the fall in tyrosyl fluorescence observed with dTdiP.

Fluorescence and polarization of DNS-nuclease: Figure 4 shows that a fall in the fluorescence intensity and polarization accompanies binding of dTdiP to DNS-nuclease. The fall in both parameters occurs over a broad pH range, and the merging of the curves in the range of pH 5 to pH 7 may represent a dissociation of

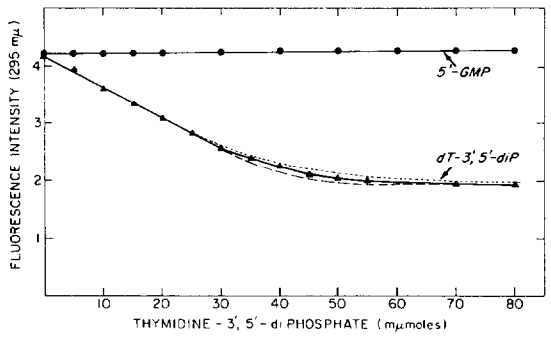

FIG. 2.—Fluorometric titration of nuclease (0.04 μmoles) with dTdiP and 5′-GMP. Studies were performed in 0.01 M Tris buffer, pH 7.0, in 0.1 M NaCl and 5 mM $CaCl_2$. The wavelengths of excitation and emission were 280 and 295 mμ. The experimental Ki is 3.6×10^{-7} (▲); for comparison, curves are presented with Ki of 1×10^{-7} (— — —) and 7×10^{-7} (- - - -).

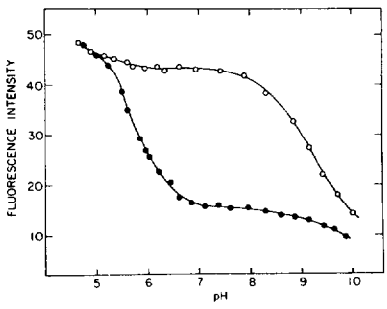

FIG. 3.—Effect of dTdiP (●) on the pH dependence of the fluorescence of nuclease (○). Solutions contained 2.6 μM nuclease, 3.7 μM dTdiP, and 5 mM $CaCl_2$ in 0.1 M NaCl. The wavelengths of excitation and emission were 280 and 295 mμ.

the protein-inhibitor complex similar to that observed for tyrosyl fluorescence (Fig. 2). The polarization depends on the lifetime of the excited state (τ) of the DNS-nuclease. A value of 13 nanosec was found which remained constant, within experimental error, at pH 4, 7.5, or 10, in the presence or absence of dTdiP.

The increase in fluorescence and polarization occurring at alkaline pH (Fig. 4) is shifted to higher pH values by almost one pH unit in the presence of nucleotide. This probably represents a stabilization by the nucleotide of the structure prevailing at neutral pH. At these high pH values, studies of the fluorescence of unconjugated nuclease are difficult to interpret because the ionization of tyrosine results in loss of its fluorescence and in the quenching of tryptophan emission due to radiationless energy transfer between tryptophan and ionized tyrosyl residues.[16]

The rotational relaxation time of conjugated nuclease was determined at pH 7.5 from a plot of $1/P$ against T/η (Fig. 5). A linear dependence was observed between 9° and 35° in the presence and absence of dTdiP. A value of P_0, obtained in 90 per cent glycerol and 10°, was nearly the same as that found by extrapolating the plots of Figure 5 to $T/\eta = 0$. The fall in polarization due to the nucleotide, although quite small, is confirmed over the entire temperature range studied. The computed relaxation times, ρ_h^{25}, in the absence and presence of dTdiP, were 17.7 and 15.6 nanosec, respectively. The smaller relaxation time observed in the presence of nucleotide might indicate a greater degree of flexibility or unfolding of the chains in the nuclease-nucleotide complex. However, the value of ρ_h^{25}/ρ_0^{25} is 1.61 for nuclease in the absence and 1.42 in the presence of the nucleotide. Both values, being greater than 1.0, indicate that the internal flexibility of either complex is negligibly small. It has been estimated from the frictional ratio that the axial ratio (a/b) of native nuclease at neutral pH is 3.7.[14] Weber[17] has computed

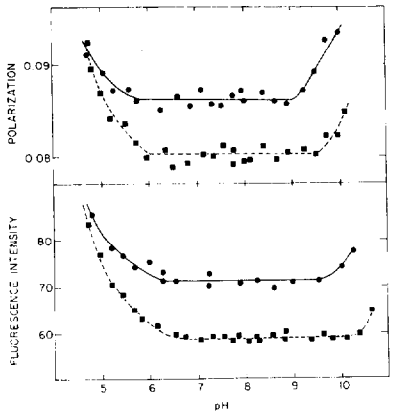

FIG. 4.—The effect of dTdiP on the pH dependence of DNS fluorescence and polarization of DNS-nuclease. Nuclease concentration was 10 μM. Titrations were done in 0.1 M NaCl, in the absence (——) and the presence (– – –) of 21 μM dTdiP and 5 mM $CaCl_2$. Samples were titrated from pH 7.2 with HCl or KOH. Wavelengths of excitation and fluorescence were 340 and 530 mμ, respectively.

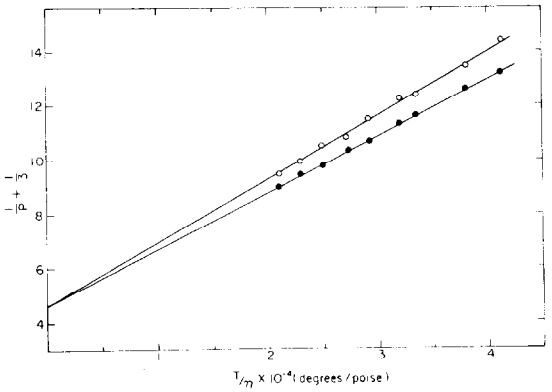

FIG. 5.—Effect of dTdiP on the dependence of polarization on T/η of DNS-nuclease. Studies were performed with 10 μM nuclease in 0.01 M Tris buffer, pH 7.5, 0.1 M NaCl, in the absence (●) and presence (○) of 14 μM dTdiP and 5 mM $CaCl_2$.

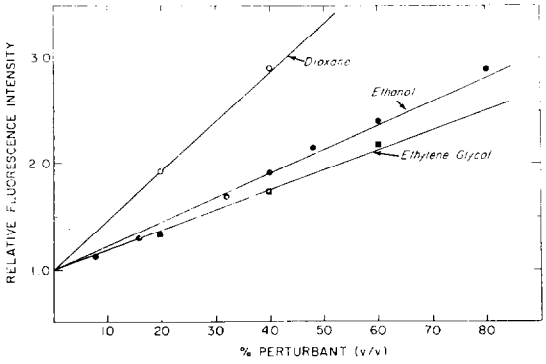

FIG. 6.—Effect of dioxane, ethanol, and ethylene glycol on the fluorescence intensity of *N*-acetyl tyrosinamide. Solutions contained 0.1 *M* NaCl and 0.01 *M* Tris buffer, pH 7.5. The wavelengths of excitation were 280 and 295 mμ.

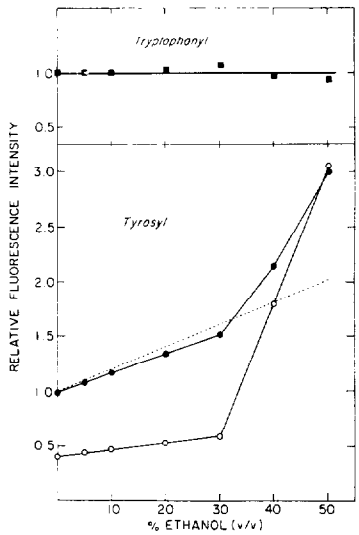

FIG. 7.—The effect of ethanol on the tyrosyl (*lower*) and tryptophanyl (*upper*) fluorescence of the nuclease. Solutions contained nuclease (8 μM) in 0.01 *M* Tris buffer, pH 7.5, 0.1 *M* NaCl, and the indicated amount of ethanol in the absence (●,■) and presence (○,□) of dTdiP (12 μM) and $CaCl_2$ (5 mM). The wavelength of excitation was 280 mμ. The tyrosyl and tryptophanyl fluorescence was measured at 295 and 360 mμ, respectively. The effect of ethanol on the fluorescence intensity of acetyl tyrosinamide is also shown for comparison (*dashed line*).

that a rigid ellipsoid of revolution of constant mass having an axial ratio of 3.7 should have a ρ_h/ρ_0 of about 1.60, and that a small decrease in ratio would lead to a slight fall in ρ_h/ρ_0. The data presented for the nuclease are therefore consistent with a slight decrease in axial ratio of nuclease coincident with the binding of nucleotide to its active site.

Solvent perturbation of nuclease fluorescence: The effect of several levels of organic solvents on the fluorescence of acetyl tyrosinamide is shown in Figure 6. A linear increase was observed for all mixed solvents in the range studied. These results are different from those reported with phenol and anisole where very little effect of reduced dielectric constant[5] has been reported.

The enhancement of tyrosyl fluorescence by solvents of low polarity might be useful as a structural probe comparable to that proposed by Herskovits and Laskowski for tyrosyl absorption.[18] Increasing ethanol concentration to 30 per cent produces a linear increase in tyrosyl fluorescence of the nuclease which is almost as large as found with acetyl tyrosinamide. In contrast, almost no effect was observed on tryptophanyl fluorescence (Fig. 7). Thirty per cent ethanol results in a 50 per cent increase in tyrosyl fluorescence of the nuclease when it is combined with nucleotide or when free. These data suggest that almost all the tyrosyl groups, except those quenched by the nucleotide, are exposed to the solvent both in the presence and absence of inhibitor. Since several tyrosyl groups are blocked by the inhibitor, the 60 per cent fall in fluorescence produced by the nucleotide represents the complete quenching of the groups that are buried since they escape completely the perturbing effects of the organic solvent.

Almost no change in tryptophanyl fluorescence of the nuclease occurs in 30

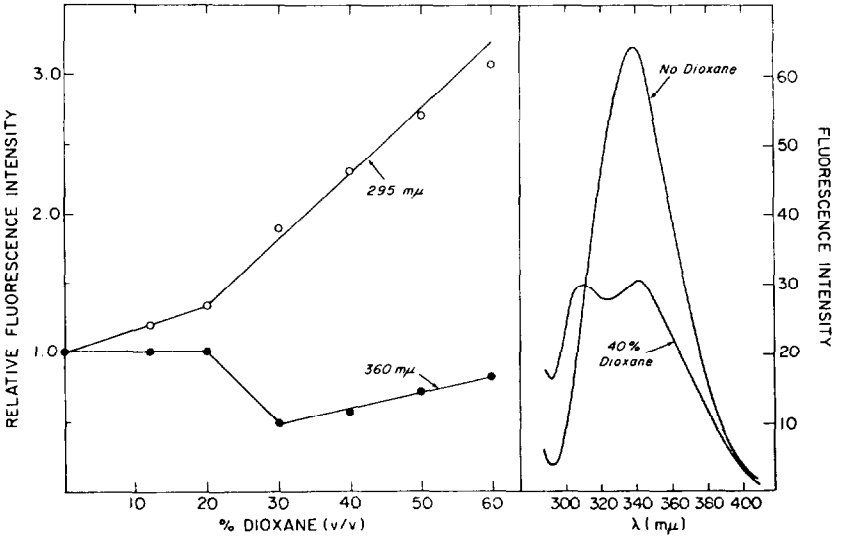

FIG. 8.—(*Left*) The effect of dioxane on the tyrosyl (○) and tryptophanyl (●) fluorescence of nuclease and the indicated amount of dioxane. (*Right*) The fluorescence spectra of nuclease in buffer and in 40% dioxane. Solutions contained nuclease (8 μM) in 0.01 *M* Tris buffer, pH 7.5, 0.1 *M* NaCl. The wavelength of excitation was 280 mμ.

per cent ethyl alcohol (Fig. 7), 20 per cent dioxane (Fig. 8), or 40 per cent ethylene glycol. Since low-molecular-weight tryptophanyl compounds show significant increases in these solvents,[4] it seems clear that the single tryptophanyl residue is almost completely buried in the native enzyme, and that the enzyme is stable in these solvents.

At higher concentrations of dioxane, the linear dependence of fluorescence is no longer observed. The tyrosyl fluorescence increases more steeply while that of tryptophan first falls by 50 per cent and then increases gradually. As a result of these changes, the single peak fluorescence spectrum observed in aqueous solution shows two peaks in 40 per cent dioxane with the contribution of each chromophore clearly evident. The slopes of the increase in tyrosyl and tryptophanyl fluorescence between 30 and 60 per cent dioxane is about that observed with model compounds and indicates a high degree of exposure.

In 50 per cent ethyl alcohol, the fluorescence intensity of the tyrosyl groups is the same in the presence or absence of inhibitor (see Fig. 7). Apparently the denaturation that occurs between 30 and 50 per cent ethyl alcohol causes complete dissociation of dTdiP from the nuclease.

Discussion.—The binding of inhibitor, dTdiP, produces an appreciable red shift in tyrosine absorption.[15] Solvent perturbation difference absorption spectra with either ethylene glycol or glycerol show a major decrease in the exposure of tyrosyl groups when the inhibitor is bound.[15] Both types of data indicate a change in the environment of the tyrosyl residues and can be accounted for by the displacement of water molecules from the vicinity of some of the tyrosyl residues.

The inhibitor also modifies the quantum yield of the tyrosyl groups by quenching almost two thirds of their fluorescence. This result cannot be explained solely by

the elimination of water from their environment since less polar solvents increase the quantum yield as seen in studies with the model compound, acetyl tyrosinamide. It has also been shown that three to four of the seven tyrosyl residues of the enzyme become unreactive to acetyl imidazole in the presence of inhibitor.[15] It has been estimated, by neutral solvent perturbation studies on the enzyme absorption spectrum, that a similar number of residues are made solvent-inaccessible by the inhibitor. If we assume that all the tyrosyl residues are approximately equally fluorescent, then the fall in fluorescence would be compatible with the complete quenching of the groups that are masked by the inhibitor provided the others were largely unaffected.

The influence of the inhibitor on the relaxation time of the nuclease, the red shift in tyrosyl absorption, and the quenching of tyrosyl fluorescence may be rationalized if the nucleotide fits into a groove or crevice containing several tyrosyl groups. In the binding process the enzyme undergoes a minor configurational change to accommodate the nucleotide, with the expulsion of water molecules formerly bound to groups in the active site. If the tyrosyl groups in the site are completely quenched, it is reasonable to assume the formation of specific bonds between them and the nucleotide. Further evidence that the nucleotide-enzyme complex is more compact than the native enzyme is evident from studies showing that the inhibitor protects the enzyme to proteolytic digestion.[19]

It is instructive to our understanding of the behavior of the nuclease to note that neither its tryptophan absorption nor fluorescence is affected by the binding of inhibitor. The spectrum of the enzyme is largely determined by the fluorescence of its single tryptophanyl residue though the tyrosyl groups absorb the major part of the exciting radiation. The environment of the tryptophan residue, which is inaccessible to the solvent in the absence or presence of inhibitor, appears to be unaffected by binding. Consequently, if a configurational change occurs, not all of the molecule is involved in this alteration in structure. It should be noted that structural effects produced either by low pH (<4.5) or high concentrations of dioxane ($\sim 40\%$) result in changes in quantum yields of both chromophores. The quantum yield of tyrosine increases several-fold in 40 per cent dioxane, while that of tryptophan diminishes significantly. Evidently the reaction with the inhibitor is not related to those changes produced by acid or dioxane denaturation.

In 40 per cent dioxane the fluorescence spectrum of the enzyme shows two peaks corresponding to the two chromophores (Fig. 8). A red shift in tryptophanyl fluorescence occurs from 338 to 345 mμ representing a normalization of its behavior relative to that of tryptophan in aqueous solution. The tyrosine peak of the enzyme is located at 310 mμ in 40 per cent dioxane. In the acid denaturation of the nuclease the fluorescence spectrum is very similar to that observed at neutral pH in 40 per cent dioxane. Evidently either method of disorganizing the protein leads to similar changes in the fluorescence yields of the chromophores.

Summary.—The binding of dTdiP to staphylococcal nuclease results in a marked quenching of tyrosyl fluorescence which can be interpreted as specific interaction of the nucleotide with several tyrosyl groups in the active site of the enzyme. In contrast to the large decrease in tyrosyl fluorescence, no change occurs in tryptophanyl fluorescence. A small fall in the polarization of fluorescence of DNS-conjugated nuclease indicates that a small configurational change accompanies the

binding of the nucleotide to the protein. The effect of the inhibitor on the degree of exposure of the tyrosyl and tryptophanyl groups has been evaluated from the influence of organic solvents on the fluorescence intensity of these fluorochromes.

* Special postdoctoral fellow, U.S. Public Health Service, National Institute of Arthritis and Metabolic Diseases.

[1] Edelhoch, H., and R. F. Steiner, in *Electronic Aspects of Biochemistry*, ed. B. Pullman (New York: Academic Press, 1964), p. 7.

[2] Weber, G., and F. W. J. Teale, in *The Proteins*, ed. H. Neurath (New York: Academic Press, 1965), vol. 3, p. 445.

[3] Cowgill, R. W., *Biochim. Biophys. Acta*, **112,** 550 (1966).

[4] Steiner, R. F., R. E. Lippoldt, H. Edelhoch, and V. Frattali, *Biopolymers, Symp.*, **1,** 355 (1964).

[5] Weber, G., and K. Rosenheck, *Biopolymers, Symp.*, **1,** 333 (1964).

[6] Cuatrecasas, P., S. Fuchs, and C. B. Anfinsen, *J. Biol. Chem.*, **242,** 1541 (1967).

[7] *Ibid.*, p. 3063.

[8] Abbreviations: dTdiP = deoxythymidine-3′,5′-diphosphate; DNS = 1-dimethylamino naphthalene-5-sulfonyl chloride or derivative with enzyme; 5′-GMP = 5′-guanosine monophosphate; 5′-dTMP = 5′-deoxythymidine monophosphate.

[9] Fuchs, S., P. Cuatrecasas, and C. B. Anfinsen, *J. Biol. Chem.*, in press.

[10] Taniuchi, H., C. B. Anfinsen, and A. Sodja, *J. Biol. Chem.*, in press.

[11] Weber, G., *Biochem. J.*, **51,** 145, 155 (1952).

[12] Steiner, R. F., and H. Edelhoch, *Chem. Rev.*, **62,** 457 (1962).

[13] Chen, R. F., G. G. Vurek, and N. Alexander, *Science*, **156,** 949 (1967).

[14] Heins, J. N., J. R. Suriano, H. Taniuchi, and C. B. Anfinsen, *J. Biol. Chem.*, **242,** 1016 (1967).

[15] Cuatrecasas, P., S. Fuchs, and C. B. Anfinsen, *J. Biol. Chem.*, in press.

[16] Steiner, R. F., and H. Edelhoch, *Biochem. Biophys. Acta*, **66,** 341 (1963).

[17] Weber, G., *Advan. Protein Chem.*, **8,** 415 (1953).

[18] Herskovits, T. T., and M. Laskowski, *J. Biol. Chem.*, **237,** 2481, 3418 (1962).

[19] Taniuchi, H., C. B. Anfinsen, and A. Sodja, these PROCEEDINGS, **58,** 1235 (1967).